Overview of studies comparing human normal cartilage with minimal and advanced osteoarthritic cartilage

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Received on July 30, 2004; accepted in revised form on January 4, 2005.

Clin Exp Rheumatol 2005; 23: 103-112.

Key words: Osteoarthritis, chondrocyte, gene expression, cartilage.

ABSTRACT

A major area under study in the osteoarthritis (OA) research field is the characterization of specific molecular and biochemical changes that distinguish advanced diseased cartilage from less involved or normal tissue. This information is important to better define the pathogenic mechanisms that are operating during OA progression and to identify disease-specific markers. This review describes recent studies that have addressed changes in chondrocyte gene expression, proliferation, and apoptosis in "experimental" (more advanced OA cartilage) versus "control" (less involved or non-OA cartilage). Included is a comprehensive listing of recently published studies in this area with general findings. The review also includes a discussion of study design and the strengths and weaknesses of the various approaches. In addition, specific strategies to deal with some of the important issues are discussed. One particular model utilizing minimal and advanced OA cartilage obtained from the same patient is described in more detail.

Introduction

Osteoarthritis (OA) is a disease of diarthrodial joints that involves bone, cartilage, and soft tissue. Both the initiation and progression of OA is multifactorial with aging, hormonal, genetic, and mechanical components involved (1). The major risk factor for developing OAis age with other risk factors including obesity and trauma (2,3). Overall, degenerative changes in the cartilage are progressive although the rate of progression will vary in specific locations of a given joint depending on a variety of factors. In addition, certain regions of the joint surface may actually display a reparative response, for example, leading to osteophyte formation (4, 5). This fact has led to the consistent observation that the degree of cartilage degeneration in human OA will vary widely within a given joint, for example, the knee, even when the overall disease is very advanced.

Therefore, studies involving human patients rather than animal models are potentially the most relevant. In fact, a variety of model systems have been described that attempt to compare matrix and cellular differences between osteoarthritic and normal cartilage using human material (Tables I and II). This list is not intended to be all-inclusive, but instead to provide an overview of the main types of comparisons that have been carried out. It is clear from these studies that multiple strategies are employed to try and establish that changes are due to disease-related factors.

The following review will present an overview of the main model systems that have been studied and discuss some of the strengths and weaknesses of each. In addition, some general findings from these studies regarding differences between the biochemistry and molecular profile of so-called normal and OA cartilage will be discussed. This review will focus on changes involving the chondrocytes including gene expression profiles, proliferation, and apoptosis. Alterations in the cartilage matrix will not be directly considered. Certain general strategies for dealing with some of the important considerations are presented. Finally, a sampling protocol for studying the phenotype of chondrocytes in advanced versus minimally involved OAcartilage on a patient-matched basis will be presented as one example of a patient-matched study.

Expected changes in advanced OA cartilage versus minimally-involved or normal tissue

The overall goal of any comparative study of disease progression is to establish changes that are related to a partic-

Table I. Representative list of studies comparing normal and OAcartilage gene expression.

Investigator	Sample characterization	Endpoint
Pullig *et al*., 1999 [18]	OAtissue from patients with primary OAcompared to control cartilage obtained at autopsy	Collagen VI expression
Pullig *et al*., 2000 [19]	Compared normal tissue obtained at autopsy within 18 hours of death with OAtissue obtained from joint arthroplasty (samples not age-matched)	Osteopontin expression
Pullig *et al*., 2002 [22]	Similar sample set as described for Pullig *et al*., 2000	Matrilin-3 expression
Kumar *et al*., 2001 [23]	Compared normal and OAcartilage both obtained at either surgery or autopsy and pooled the samples. Age or joint surface information was not provided	Expressed sequence tag analysis looking at differential expression
Martin *et al*., 2001 [24]	Compared normal cartilage obtained from femoral neck fracture repair or foot amputation (ankle) with OAcartilage obtained from various joints at arthroplasty (samples not age-matched)	Expression pattern of extracellular matrix genes
Steck *et al*., 2002 [25]	Compared cartilage obtained fromvarious joints obtained from joint arthroplasty with normal cartilage obtained from various joints from crime victims and tumor resection procedures (samples not age-matched)	PCR-based subtractive hybridization to find unique genes
Aigner *et al*., 2001 [26]	Compared OAcartilage obtained from joint arthroplasty with control cartilage obtained at autopsy	cDNAarray and real time PRCanalysis of collagen and MMPexpression
Bau *et al*., 2002 [27]	OAcartilage obtained from joint arthroplasty with control cartilage cartilage obtained post-mortem within 24 hours of death	MMPgene expression with real time PCR
Bau *et al*., 2002 [28]	Similar sample set as described above for Bau *et al.*	Smad expression
Gebhard *et al*., 2003 [29]	Compared normal tissue and early degenerative tissue obtained from autopsy material within 48 hours of death with late stage OAtissue obtained from joint arthroplasty (Samples not age matched)	Expression level of mRNAs coding for various collagens
Aigner *et al*., 2003 [30]	Similar sample set as described for Gebhard *et al*.	Array analysis and real time PCR focusing on MMPgene expression
Bock *et al*., 2001 [31]	Comparison of lesion and intact areas of femoral cartilage obtained from joint arthroplasty	Decorin and biglycan expression
Iannone *et al*., 2001 [32]	Comparison of cartilage from regions with minimal involvement to areas with more advanced disease and non-OAcartilage	IL-10/IL-10R
Iannone *et al*., 2002 [33]	Similar sample set as above	NGF/TrkA

ular disease state compared to another disease state or that are different from normal tissue. In the case of OA, and focusing just on cartilage, various changes might be anticipated in the resident cell population, the extracelluar matrix or both. This is because the resident chondrocytes, while displaying relative low metabolic activity in stable adult articular cartilage (6) are, in fact, a pleiotropic cell type capable of multiple phenotypic pathways. One or more of these pathways may dominate for a particular population of chondrocytes in a particular region during a specific period of pre-natal development or age of the adult organism. For example, high level expression of genes coding for numerous cartilage matrix proteins such as collagen II and aggrecan is typical of chondrocytes during the initial formation of the hyaline cartilage anlagen of the developing skeleton (7). In comparison, the expression of collagen X and specific matrix metalloproteinases such as MMP-13 are characteristic of chondrocytes undergoing hypertrophy in the terminal zones of the growth plates (8). This hypertrophic zone of the growth plate also includes chondrocytes that are undergoing apoptosis during the replacement of the cartilage by bone (9). Animal studies suggest that increased apoptosis in articular cartilage may be one reason for the decreased cellularity that has been reported during aging (10). In addition, the loss of responsiveness of aged chondrocytes to growth factors has been reported in both animal and human studies (11) and may represent another age-associated change in cartilage that is distinct from OAbut possibly predisposes the cartilage to development of disease. The fact that specific age-associated changes in articular chondrocytes have been reported suggests that comparative studies examining normal and diseased cartilage should take age-differences of the source tissue into account.

Other anticipated changes in the molecular phenotype of chondrocytes during OA progression are predicted from the altered histology and biochemistry that has been documented in OA. For example, the loss of structural integrity of the articular cartilage with advancing OA suggests that degredative enzymes are involved. In fact, the expression of several MMPs by articular chondrocytes is well documented (12). However, the precise molecular phenotype of human articular chondrocytes with

Table II. Studies comparing normal and OAcartilage proliferation and apoptosis.

Investigator	Sample characterization	Endpoint
Hashimoto *et al*., 1998 [32]	OAtissue from arthroplasty compared to non-OAtissue from autopsy and tissue banks	Apoptosis
Kouri *et al*., 2000 [33]	OAtissue from arthroplasty compared to normal tissue from autopsy material (within 3 hours of death)	Apoptosis
Yatsugi *et al*., 2000 [34]	Compared OAcartilage obtained from hip arthroplasty with normal cartilage from surgery for femoral neck fracture repair (samples not age-matched)	Expression of apoptosis genes
Kim *et al*., 2000 [35]	OAtissue from arthroplasty compared to normal tissue from autopsy material (the post-mortem delay was not defined)	Apoptosis
Aigner *et al*., 2001 [36]	Post-mortem tissue obtained within 24 hours of death for normal samples compared to OAtissue obtained from joint arthroplasty	Apoptosis
Cheng *et al*., 2004 [37]	Compared OAcartilage obtained from hip arthroplasty with normal cartilage from surgery for femoral neck fracture repair (samples age-matched)	Expression of a protein marker of apoptosis
Sharif *et al*., 2004 [38]	Compared OAcartilage obtained from hip arthroplasty with normal cartilage from surgery for femoral neck fracture repair (samples age-matched)	Expression of TUNEL+ cells and caspase-3
Kusuzaki *et al.*, 2001 [37]	Compared OAcartilage obtained during joint arthroplasty with control cartilage obtained at autopsy from a variety of joints	Chondrocyte proliferation
Lapadula *et al*., 1995 [42]	Comparison of cartilage from regions with minimal involvement to areas with more advanced disease and non-OAcartilage disease and non-OAcartilage	Cell-cycle CD10, CD26
Lapadula *et al*., 1997 [43]	Similar sample set as above	Cell-cycle, integrin

regard to MMP gene expression during different stages of OA disease progression is still not fully established.

Additional chondrocyte phenotypes which may or may not be related to the pathogenesis of OA have been described. For example, "modulation" or "dedifferentiation" refers to a switch in the pattern of gene expression to one that is more typical of fibroblasts or mesenchymal cell precursors of chondrocytes (13-15). Finally, chondrocytes may also display a classic senescent phenotype *in vivo* based on expression of the senescence-associated beta-galactosidase marker (16,17). It is important to note that in intact cartilage there may be heterogeneous populations of chondrocytes resulting in a more complicated gene expression profile than might be predicted from any of one of these individual classifications. The next section will outline some of the model systems that have been studied in order to help define the molecular phenotype of the chondrocyte in human OA. It will be clear that each of these approaches has both strengths and weaknesses.

General overview of model systems

The recent literature contains numerous reports of changes in chondrocytes residing in human OA tissue compared to “normal” tissue. Clearly the reported changes must be considered in light of how the “normal” and OA tissue were obtained and how the comparisons were made. In this review, the term "normal" will be used to describe cartilage that is used as the control tissue for comparison to clearly diseased, i.e. experimental, cartilage. Some examples of cartilage sources include: 1) Studies comparing OA tissue obtained at joint arthroplasty with normal tissue obtained from young donors; 2) Studies comparing OA and normal tissue obtained from different joints; 3) Studies comparing OA tissue obtained from joint arthoplasty with normal tissue obtained at autopsy or from cadavers. These samples may or may not be age-matched and have varying degrees of histological confirmation.

Summary of published research comparing normal and OA cartilage focusing on gene expression patterns

The following studies are representative of a larger body of research that is directed at comparing the expression of genes coding for various matrix proteins by chondrocytes residing in normal or OAcartilage. These studies were included because they are relatively recent and because the model systems that were studied are relatively well defined. One study compared cartilage obtained from patients with primary OA undergoing total knee replacement with samples obtained from individuals at the time of autopsy (18). The general finding was that both normal and OA cartilage expressed type VI collagen in specific zones and that the overall expression of this collagen was increased in the OA cartilage. The same group (19) compared the expression of osteopontin in normal and OAcartilage. Normal cartilage was obtained at autopsy within 18 hours of death and OAtissue was obtained from joint arthroplasty. The samples were not age-matched. Osteopontin is a sulfated phosphoprotein that binds to cells and extracellular matrix (20) and has been reported in the late hypertrophic chondrocytes of the growth plate. No evidence of osteopontin expression was observed at the mRNAor protein level in normal cartilage. In comparison, chondrocytes expressing osteopontin were detected in the upper deep zone of OA cartilage. This finding was verified by western blot analysis. The investigators suggest that expression of osteopontin in OA

may represent a shift of the phenotype of the chondrocytes to that found in the late epiphyseal growth plate. This finding supports other work from the same laboratory that demonstrated increased osteocalcin and collagen X expression in OAcartilage compared to normal cartilage (21). Finally, using similar sample groups, these investigators found that matrilin-3, a recently described extracellular matrix protein, showed enhanced expression in OAcompared to normal (22).

Several additional laboratories have examined general and specific gene expression patterns in normal and OA cartilage. Work by Kumar *et al*. (23), utilized cDNA libraries from human osteoarthritic cartilage obtained from patients undergoing knee replacement surgery and from normal cartilage obtained from individuals at the time of autopsy. The degree of degeneration was assessed by standard histological criteria and no information was provided as to the age of the patients. In order to provide enough material for cDNA library construction RNA samples were pooled. The investigators provide informative tables listing the known proteins identified in the cartilage samples as well as the identification of proteins that were not previously reported to be expressed in cartilage. In addtion, information is provided as to the most abundant genes expressed in both normal and OAcartilage.

Another study compared normal cartilage obtained from femoral neck fracture repair or foot amputations (ankle cartilage) with OA cartilage obtained from various joints at arthoplasty (24). The samples were not age-matched and were divided into control or OA according to clinical evaluation as well as histological and biochemical criteria. Real time PCR primers were designed for collagens I, II, and X, aggrecan, versican, osteopontin, and osteocalcin. The expression level of these matrix genes was normalized to GAPDH. With regard to individual gene expression levels, only osteopontin was found to be elevated in OAcompared to control cartilage. This result supported the finding of Pullig *et al*. (19). Also, the ratio of collagen II to collagen I and aggrecan to versican were both found to be higher in control cartilage (27.0 and 7.6-fold respectively) suggesting a loss of differentiated phenotype in chondrocytes associated with OA cartilage. The investigators make the interesting observation that ratios of gene expression patterns may be a more sensitive indicator of differences between control and OA chondrocytes than looking at individual genes.

A different approach was reported to examine general patterns of gene expression in normal and OA (25). The aim of this study was to identify differences in gene expression between normal and OAcartilage using a combination of cDNA representational difference analysis (cDNA-RDA) and real time PCR to verify the differences. As indicated previously, it is very important to define the source of the samples. In this case, OA cartilage was obtained from the tibial plateaus of patients undergoing total knee or hip arthroplasty. Normal cartilage was collected from two crime victim patients and patients undergoing amputation for tumor resection. There is no indication of patient age or histological verification of the disease state of the tissue. The basic finding of this study was an up-regulation of YKL-39 mRNAin OAcartilage versus normal cartilage but no up-regulation of a homologous molecule, YKL-40 (chitinase 3-like 1). In fact, YKL-40 has been reported to be upregulated in synovial fluid and serum from patients with joint disease but this study suggests that the source of this protein may be synovial cells and not chondrocytes. The up-regulation of YKL-39 which was initially observed by subtractive hybridization was confirmed by array analysis and by real-time PCR. The significance of YKL-39 upregulation to the pathogenesis of OAis not known.

A series of studies from one laboratory focused on several important endpoints relevant to the pathogenesis of OA. This group primarily utilizes OA cartilage obtained at the time of joint arthroplasty with control cartilage obtained at autopsy. The post-mortem delay is reported to be anywhere from 24-48 hours for the control tissue. In one study (26) the major findings include strong expression of collagen II and VI in late stage OA specimens and increased fibronectin expression in early stage OA. MMP-3 was reported to be down-regulated and MMPs-2 and –11 were both up-regulated in late-stage disease. Utilizing a similar sample set these investigators reported that MMP-13 and ADAM-TS5 were the major collagenase and aggrecanase, respectively, in OA cartilage (27). MMP-1 was expressed at a very low level in all conditions and MMP-3 was the most strongly expressed MMP but was down-regulated in late-stage OA(27). In addition, these investigators have found that BMP-receptor Smads 1, 5, and 8 as well as common Smad (C-Smad) 4 are expressed in both human normal and OAarticular chondrocytes. They report no major up-regulation or down-regulation of any of the Smads in OA(28).

Two publications from this group compare advanced OA cartilage obtained from joint arthoplasty and normal and minimal OA cartilage obtained from post-mortem specimens within 48 hours of death. The minimal and advanced OA specimens were age-matched but the normal cartilage was obtained from younger individuals. One study focused on collagen gene expression using quantitative real time PCR (29). The overall findings indicated an increase in the mRNAcoding for the various collagen types in advanced OA compared to early degenerative and normal cartilage. In addition, the ratios of mRNA coding for different collagen types did not suggest a shift toward a more undifferentiated phenotype. This finding is somewhat in contradiction to the results described above by Pullig *et al*. (19) that suggested a decrease in the ratio of aggrecan to versican and collagen II to collagen I in OAsamples compared to normal. The concern regarding the study by Gebhard *et al*. is the fact that samples representing the normal and early degenerative OA categories were collected over a 48 post-mortem time period. Therefore it is difficult to be certain that the differences in relative expression of specific mRNA transcripts might not have been influenced by this data collection paradigm. However, the increased expression of collagen m-

RNAs does support the overall hypothesis that OA progression can be associated with an anabolic or repairative response.

A recent publication from this group (30) utilized normal femoral condylar cartilage from donors at autopsy within 48 hours of death with an age range of 39-72 years old. The OA cartilage was obtained from patients undergoing total knee replacement surgery with an age range of 61-73 years old. The normal and OA samples were compared by cDNA array analysis to determine differential steady-state levels of specific mRNA transcripts. Clearly, while the work is potentially interesting, the issues of post-mortem changes, non-age matched samples, and use of different joints must be considered when evaluating the significance of the results. The investigators report down-regulation of MMP-3 in late stage OA. Neither MMP-1 nor MMP-8 mRNA was detected in any sample, and MMP-13 was found to be expressed at higher levels in OA cartilage compared to normal. The expression patterns were confirmed by Real Time PCR. A variety of other genes were found to be differentially expressed in normal versus OA cartilage.

An interesting sample set was compared by Bock *et al*. (31). This study analyzed biglycan and decorin expression in a macroscopically intact area, an adjoining area, and a lesioned area from knee joints of patients with OA who were undergoing joint arthroplasty. The major finding was an increase in the expression of both proteoglycans in a specific population of chondrocytes in areas of cartilage adjoining the main defects.

The approach of comparing the lowest and highest degree of OA severity within single patients has been reported in additional publications (32, 33) and has resulted in significant findings. For example, it was found that nerve growth factor (NGF) protein expression level was low in normal chondrocytes and increased progressively in minimal and more advanced diseased cartilage (33). The expression of the NGF receptor (TrkA) showed a similar pattern suggesting that NGF may play a role in the pathogenesis of OA. Further, IL-10 was also expressed at progressively higher levels in more advanced diseased cartilage compared to minimal OA or non-OA cartilage (32). These studies are significant because they utilize, in part, intra-patient comparisons which control for many of the potential variables and they point to specific molecular mechanisms that may be operating during OAprogression.

Summary of published research comparing normal and OA cartilage focusing on chondrocyte proliferation and apoptosis

Several studies have reported either direct or indirect evidence of increased apoptosis associated with human OA (34-37,39,40). Utilizing several endpoints, Hashimoto *et al*. (34) analyzed apoptotic cells in OAcartilage from patients undergoing arthroplasty compared to non-OA cartilage obtained at autopsy or from tissue banks. These investigators reported that 22.3% of cells were undergoing apoptosis in the OA cartilage compared to 4.8% of chondrocytes in the non-OA cartilage. Further, this study reported the important finding that areas of cartilage that contained apoptotic chondrocytes also showed loss of proteoglycan. A separate study (35) found that the presence of apoptotic chondrocytes correlated with factors involved in calcification and concluded that apoptosis is involved in the abnormal calcification observed in OA cartilage. A third study (36) compared cartilage obtained at joint arthoplasty from patients with RA or OA with control articular cartilage from patients with femoral neck fracture. They reported finding apoptotic chondrocytes in cartilage with a relatively early degenerative changes and a greater number of apoptotic chondrocytes in RA than OA. In comparison few apoptotic chondrocytes were reported in control cartilage.

These morphological findings correlated with expression patterns of p53 and c-myc, which are apoptosis related proteins. Kim *et al*. (37) reported that the percentage of apoptotic cells in lesional areas of OA cartilage was significantly higher than in non-lesional areas of cartilage from the same patient, while apoptotic cells were rarely seen in normal cartilage. The comparison of lesional and non-lesional regions of cartilage from the same patient is an approach that we will expand on in the next section.

A separate study (38) utilized a similar sample set as previously reported from this group. Namely, post-mortem tissue obtained within 24 hours of death for normal cartilage compared to OAcartilage obtained from joint arthroplasty. They report no evidence of apoptotic chondrocytes in aging or OA cartilage except in the calcified layer. They do report an increase in empty lacunae in both late-stage OA and during aging of normal cartilage. They conclude that apoptotic cell death is not a widespread phenomenon in aging or OA cartilage. However, the most recent studies examining this question do find evidence for increased apoptosis in human OA. For example, the expression of PDCD-5, a novel apoptosis related protein was found to be increased in chondrocytes isolated from osteoarthritic cartilage versus non-OA cartilage (39). The OA cartilage was obtained from patients undergoing knee or hip arthroplasty and the non-OA cartilage was obtained from age-matched patients requiring arthroplasty due to osteoporotic femoral neck fracture. A similar sample set was used to show increased TUNEL-postive cells and increased caspase-3 expression, both markers of apoptosis, in OAversus non-OAcartilage (40).

Finally, a published study (41) reported on using DNAcytofluorometry to measure cell kinetics from normal aging cartilage and OA cartilage. The normal aging cartilage was obtained during autopsy and was isolated from a variety of joints. The OA cartilage was obtained during total knee or total hip replacement and was divided into mild, moderate, severe or regenerative tissue based on Mankin criteria. This study makes reasonable comparisons between groups, considering that the focus is on an analysis of chondrocyte proliferative activities and not gene expression profiles. The data suggests that aging cartilage contains chondrocytes which are in G0 but are polyploid while in-

creasing severity of OA is associated with an increased percentage of cells in the S- and G2-phases of the cell cycle. One possible confounding factor is the fact that the samples were taken from multiple joint sites, but the study does divide the analysis into weight bearing and non-weight bearing regions which were found to have a similar pattern.

The finding described above is actually confirmatory for previous reports using a study design that compared different regions within the same patient with varying degrees of disease severity and also included the analysis of non-OA cartilage (42,43). These investigators found that the percentage of cells in G0 was higher in anatomically normal cartilage and that the S-phase of the cell cycle prevailed in cells isolated from the most damaged zone. It was further hypothesized that the changes in cell cycle profiles were related to a demonstrated inverse correlation between beta-1 integrin expression and lesion severity and/or decreased expression of membrane bound peptidases CD10 and CD26 in the maximal load zone.

Consideration of study designs

The major confounding variables in the studies reviewed in the previous section are age (19, 21, 24-26, 29, 36, 38), differing genetic backgrounds of the patients (virtually all of the reported studies), and post-mortem delay (19, 21, 26, 29). There are clearly justifications for choosing a particular comparison and there is a need for multiple, parallel approaches. While the work of these investigators is certainly important, the particular paradigm utilized in each of these studies must be taken into account when the final results are discussed. Certain factors become increasingly important if the study design is supporting a screening procedure, such as a DNA array analysis, because the final differences are only meaningful in the context of the model system that was used.

For example any study that compares normal healthy articular cartilage obtained from cadaver material to cartilage obtained from joint arthroplasty is problematic. This is especially true if the acceptable period of post-mortem delay extends out to 48 hours. It is possible, for example that some of the differences in patterns of collagen expression found in these studies are influenced by issues of message stability and/or changes in the chondrocyte phenotype after death. In general, study designs should include precise protocols for obtaining the tissue with regard to post-mortem delay and storage and for preservation of the samples, whether for RNA isolation, histology, or biochemical analysis. The possible difference between superficial and deep chondrocytes should be noted, as this may influence the final results depending on how the tissue was obtained, One approach is to always obtain full-thickness cartilage samples and utilize histological confirmation of what the cell populations may be lost with disease progression.

Another issue that needs consideration is the possibility of variations in loading parameters between cartilage sampled from different locations. From a practical standpoint, increased load will often be associated with more advanced disease. This is evident from individuals with valgus versus varus deformities in which increased subchondral bone mineral density is observed in the lateral versus medial compartment of the knee respectively (44). In spite of this relationship, it is still reasonable to look for unique gene expression profiles between chondrocytes residing in OA cartilage with varying degrees of disease severity, since this is an indication of how the cell response to the increased load may drive OA progression. It is also possible to identify differential patterns of gene expression that are related to different anatomical locations, independent of load. For example, cartilage that is carefully scored as minimally involved can be isolated from femoral heads removed secondary to hip fracture and compared to a similar sample set from femoral condyles. Another site that has been used for obtaining non-involved OA cartilage is the ankle joint since this anatomical site has a lower incidence and slower progression of OA as compared to the knee (45).

Another useful control would be to compare patients with varus and valgus OAin terms of patterns of gene expression. Patients with varus deformities have predominately medial compartment advanced disease with minimal involvement of the lateral compartment and this pattern is reversed in the majority of patients with valgus deformities (44, and our unpublished data). Therefore gene expression patterns between patient-matched compartments could be compared to see if the pattern of gene expression reflects the disease severity rather than the joint compartment. Also the use of cartilage obtained from age-matched patients undergoing joint resection for various chondrosarcomas that require removal of otherwise normal articular surfaces will provide an additional basis for comparison. If the patients are age-matched then it may be possible to determine how similar the minimally involved OAcartilage is to truly non-involved articular cartilage. Gender and treatment or medication history are possible contributing factors to altered patterns of gene expression. The former is easy to control for while the latter is more difficult. Obviously, experimental approaches comparing patient-matched minimally involved versus more advanced OA cartilage would control for these variables (31-33,42,43,46,47).

Finally, the approach that is used to study the various sample sets is of critical importance. For example, there are many different approaches to identify and quantify apoptosis (48) which can lead to discrepencies in the literature. If possible, studies designed to confirm existing data in different sample sets should utilize similar endpoints for analysis. Given the limited material that is often available from human specimens, quantitative real time polymerase chain reaction (QtRT-PCR) has become a common technique for analyzing gene expression changes (49). This technique is sensitive and accurate for this purpose if all of the proper validation experiments are performed and controls are included (49). We have utilized this procedure to examine changes in chondrocyte gene expression *in vitro* and have found excellent correlation with more traditional northern an-

Table III. Scoring system.

Fibrillation 1-5 (1: minimal, 5: severe)
Chondrocyte cloning 1-4 (1: minimal, 4: pronounced)
Proteoglycan depletion (% of non-metachromatic area/total area)
Cellularity of metachromatic and non-metachromatic regions

Minimal versus advanced OAcartilage samples are obtained as described in the text. The mean scores for multiple patients are compared to determine the distinctness of the two sample sets.

Table IV. Mean scoring data of minimal versus advanced OAcartilage.

	Minimal	Advanced
Fibrillation	2.23 ± 0.68	4.86 ± 0.35***
Chondrocyte cloning	2.33 ± 0.83	3.57 ± 0.82**
% Proteoglycan depletion	23.69 ± 15.5	55.36 ± 26.16***
Cellularity		
Non-metachromatic area	29.56 ± 13.72	29.40 ± 22.01
Metachromatic area	10.69 ± 3.69	11.80 ± 3.66

Scoring data are derived from at least 13 patients in each category. Asterisks represent statistical significance at the $p < 0.01$ (**) or $p < 0.001$ (***) level.

alysis (50). Currently we are using this approach to define gene expression patterns in our minimal versus advanced OA cartilage samples (see next section). Investigators who are collecting samples for one analysis should consider the possibility that future studies with different end points may be beneficial. Therefore, archiving of quick frozen tissue for biochemical analysis and fixing and storing tissue for possible immunohistochemical studies is desirable. In the next section we present some preliminary data comparing histological, biochemical, and gene expression data in a model of minimally involved and advanced OAcartilage.

A model for analyzing patterns of chondrocyte gene expression associated with different disease severity in human osteoarthritis

Several laboratories have utilized one approach toward studying changes with less versus more severe OA that does circumvent some of the problems associated with non-age matched or non-genetic matched samples as well as post-mortem changes. In addition, this approach would control for issues such as patient history. Basically comparisons are made utilizing cartilage obtained from the same patient undergoing joint replacement in which there are varying degrees of disease progression (31-33,42,43,46,47). We have further developed this approach as described below. This model is included simply to illustrate one approach that attempts to control for some of the variables discussed previously.

Cartilage showing no evidence of gross disease is taken from larteral femoral condyles while cartilage showing degenerative changes is sampled from the medial femoral condyle within 10 mm of overt lesions. These sample pairs are obtained from multiple patients and termed minimal and advanced OA cartilage. It is important to note that the terms "minimal" and "advanced" represent designations based initially on the presence or absence of overt cartilage degeneration and both are labeled as "OA cartilage" since this tissue was clearly exposed to an active disease process. In fact, the first hypothesis tested was that minimal and advanced OA cartilage would show consistent histological differences across a variety of patients based on specific scoring criteria that reflect important pathogenic events in OA(Table III). The mean differences in these scores is shown in Table IVand indicates that the minimal and advanced OA cartilage samples are, on average, distinct with regard to fibrillation, proteoglycan depletion, and chondrocyte cloning. The typical appearance of the minimal versus advanced OAcartilage is shown in Figure 1A & B respectively. In general, minimal OAsamples showed a uniform distribution of chondrocytes with intense metachromasia except in the superficial zone and only minor or no surface irregularities. The advanced OA cartilage displayed fibrillation (clefts), loss of metachromasia indicating loss of proteoglycan and evidence of a proliferative response of chondrocytes (cloning). A biochemical assessment of protoeglycan content based on detection of chondroitin sulfate (51) showed a statistically significant decrease in the advanced versus the minimal OA cartilage (0.121 ± 0.03 ug CS/ug dry weight for minimal versus 0.07 ± 0.03 for advanced, $n = 5$).

This model will be useful for testing specific hypotheses regarding the pathogenic mechanisms involved in OA progression, as well as for carrying out more general screening studies to identify novel markers for OA progression. As an example we have analyzed the expression of a variety of genes including aggrecan and osteopontin at the mRNA level using quantitiative real time PCR. As shown in Figure 2 the steady state level of mRNA coding for osteopontin is significantly elevated in advanced OA cartilage compared to minimal OA on a patient-matched comparison. In comparison, 9 out of 11 patients showed decreased steady state levels of aggrecan mRNA in advanced versus minimal OAcartilage. These results support one of the consistent findings reviewed earlier, that osteopontin expression is upregulated in OA compared to normal cartilage (19, 24). Also, these preliminary results show good correlation between histological, biochemical, and gene expression endpoints with respect to aggrecan expression.

There are still several issues that must be taken into account with the approach described in this section and utilized by other laboratories. For example, the minimal and advanced OA cartilage is sampled from different joint compartments. As was mentioned previously, additional sample sets will be

important to further define the basis of the altered patterns of gene expression; for example, comparing valgus versus varus OA and including non-OA cartilage. This last comparison will be important for testing the hypothesis that the minimal OAcartilage is more closely related to normal cartilage than the advanced sample. However, the data shown above clearly demonstrate that chondrocytes residing in minimal OA cartilage have a distinct pattern of gene expression compared to cells residing in the more advanced tissue independent of gender, age, genetic background or patient history. It will be important to further understand the basis for this altered gene profile.

Conclusion

There is an increasing body of data on changes in chondrocyte gene expression, proliferation and apoptosis in human cartilage derived from OA and non-OA joints. It is clear that no single approach toward defining these changes is fully adequate and additional studies are needed to document and clarify published findings. In some cases consistent findings are emerging such as increased osteopontin expression associated with OA. There are also contradictory findings; for example, various studies have reported a large, minimal, or no change in apoptosis in OA cartilage compared to normal tissue. There are many possible reasons for these different findings, including the choice of the model system, the heterogeneous nature of the chondrocytes present in the cartilage and the different methods utilized for measuring apoptosis (48). Future studies using additional model systems and DNA array analysis with confirmation by real time PCR will likely define precise changes in gene expression that contribute to the pathogenesis of OA. These studies should include precise documentation of disease severity, comparison of appropriate sample sets, careful isolation, preservation, and characterization of cells and tissue, proper validation of findings with multiple endpoints and adequate patient information.

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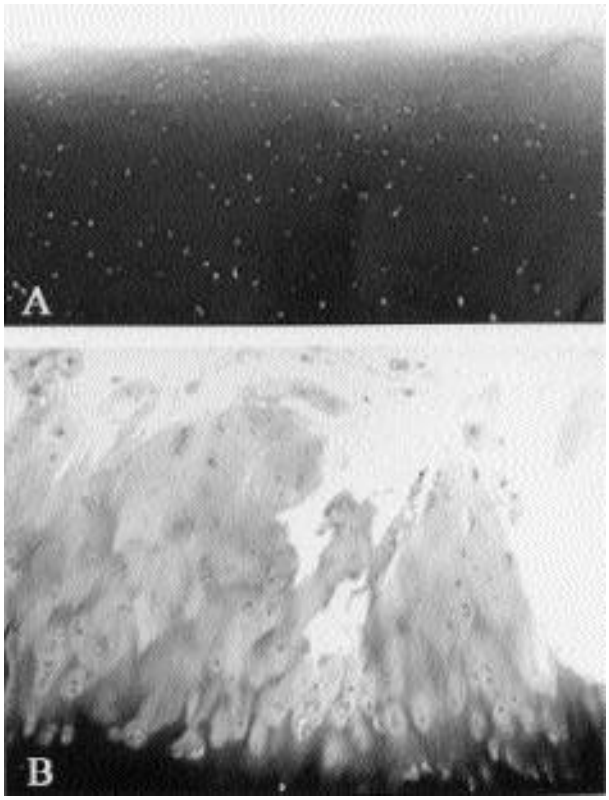

Fig. 1. Histology of a patient-matched sample of minimal and advanced OAcartilage.
The cartilage was sampled from a minimally involved and advanced disease region based on the gross criteria outlined in the text. (**A**) The minimally involved OAcartilage shows an even surface, uniform distribution of chondrocytes and intense metachromasia throughout most of the thickness of the tissue. (**B**) The advanced OA cartilage has a disrupted surface with deep clefts, loss of metachromasia, and focal chondrocyte proliferation ("cloning"). These characteristics are highly reproducible among multiple patient-matched samples.

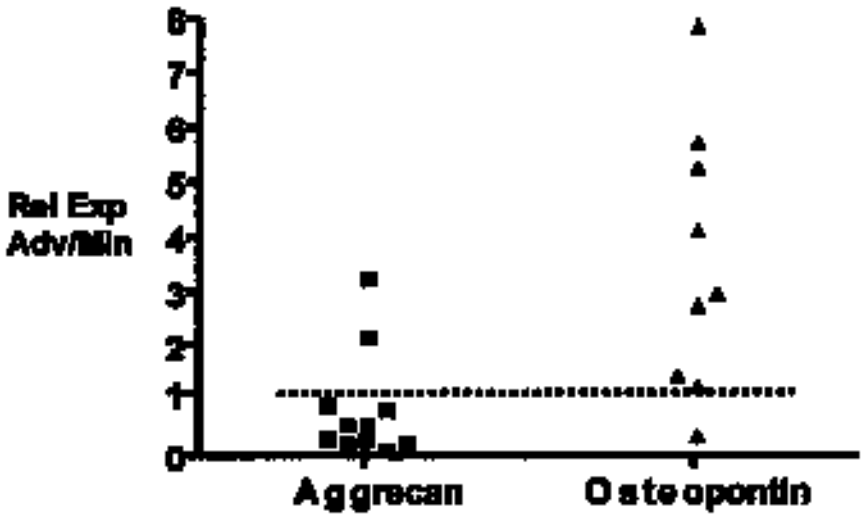

Fig. 2. Expression of osteopontin mRNAdetermined by quantitative real time PCR.
RNA was isolated from minimal and advanced OA cartilage from 9-11 patients. Following reverse transcription the cDNAwas amplified with human primers specific for aggrecan or osteopontin and the relative amounts of product determined by syber green fluorescence using the ABI Prism 7700 system. The samples were normalized to 18s ribosomal mRNAwhich was determined to express at similar levels in the different samples. The data are presented as relative expression between patient matched advanced versus minimal OAcartilage with the dashed line indicating no difference between the two regions.

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